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Glutathione-Coordinated [2Fe-2S] Cluster: A Viable Physiological substrate for Mitochondrial ABCB7 Transport

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Abstract

The glutathione-coordinated [2Fe-2S] cluster is demonstrated to be a viable and likely substrate for physiological iron-sulfur cluster transport by Atm1p, a mitochondrial ABC export protein. Flow cytometry and colorimetric assays demonstrate a quantitative methodology for study of metal translocation proteins and their proteoliposome products.

Atm1p is a yeast homolog of the ABC7-type ATP-dependent mitochondrial membrane transporter that is linked both to mitochondrial iron homeostasis,^{1–4} and the regulation of cytosolic iron concentration.^{5–8} Deletion of the human homolog leads to severe iron-sulfur cluster deficiency in the cytoplasm, but not in mitochondria.^{9–14} Key roles in cytoplasmic iron-sulfur cluster assembly have also been reported in *A. thaliana* for other Atm1 family proteins, indicating their importance in the plant kingdom,¹⁶ and a role suggested in heavy metal detoxification by a bacterial homolog of Atm1p.¹⁷ While the identity of the transporter substrate remains uncertain, glutathione (GSH) has been implicated.^{13, 14}

We have recently reported a novel water-stable glutathione complex of a [2Fe-2S] cluster core, [2Fe-2S](GS)₄^{2–}, which demonstrates reversible exchange chemistry with eukaryotic ISU-type iron-sulfur cluster scaffold proteins, and stimulates Atm1p ATPase activity – a hallmark of a transporter substrate.^{18–20} Furthermore, an amino acid substitution at the proposed substrate binding site eliminated [2Fe-2S](GS)₄^{2–} cluster binding while retaining ATPase activity.²⁰ These observations are consistent both with an essential role for glutathione in mitochondrial cluster export,²¹ and provide a ready explanation for Atm1p-associated glutathiones in recently solved crystal structures,²² as well as supporting a role for the transport of glutathione-coordinated heavy metals.¹⁷ Herein, we report the results of experiments that provide direct support for glutathione-complexed Fe-S cluster as a physiologically viable substrate for mitochondrial ABCB7-type transporters. In particular, we present evidence of the ability of Atm1p to transport [2Fe-2S](GS)₄^{2–} (as well as a fluorophore-labeled cluster adduct) by use of independent flow-cytometric methods and iron absorbance assays in a model proteoliposome complex, which also allowed quantification of cluster transport activity and determination of kinetic rate constants. These

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†Electronic Supplementary Information (ESI) available: Experimental details concerning the synthesis of proteoliposome, fluorescein-labeled cluster, cluster transport, flow cytometry experiments and iron quantitation assays.

experiments provide further support for the physiological relevance of glutathione-complexed [2Fe-2S] clusters, demonstrate the viability of such species as natural transporter substrates, and present a quantitative methodology for study of metal translocation proteins and their proteoliposome products.

Glutathione-coordinated iron-sulfur cluster is stable under physiological matrix conditions, where excess cellular glutathione prevents cluster hydrolysis. ESI mass spectrometry, combined with studies of functional group modification suggest salt-bridges to be important in glutathione tetramer formation, which creates a macrocyclic ligand that accepts the [2Fe-2S]²⁺ cluster core from the scaffold protein ISU.^{18, 19} Functional studies of Atm1p have now been conducted with protein-embedded proteoliposomes and transport monitored by a novel application of both flow cytometry and tiron-ligated absorption assays. The flow cytometer was able to both detect proteoliposomes ~400 nm in diameter and quantitate the fluorescence signals of the content inside. In the case of fluorescein-loaded liposomes, a decrease in fluorescence signal was observed throughout the cluster transport reaction, because of the inner-filter effects of iron-sulfur cluster transport into the liposome (Figure S1). The concentration of fluorescein was maintained under 1 mM, which is not concentrated enough for self-quenching to occur.²³ The synthesized proteoliposomes remain intact and not leaky, as observed in Figure 1 where control experiments demonstrated no change in emission over a period of 1 hr. The advantage of particle-specific signal measurements allow events during proteoliposome detection to be correlated with the fluorescence signal associated with the proteoliposome rather than the bulk of the solution. Moreover, the cuvette pathlength is 56-fold thinner than the conventional 10 mm cuvette found in standard fluorimeters, and the thinner cuvette design eliminates 98% of the solution noise that is not associated with the proteoliposome.

The addition of cofactor Mg-ATP provides the energy source for Atm1p to transport substrate into the proteoliposome. Flow cytometric studies of the reaction mixture containing Atm1p proteoliposome, cluster and Mg-ATP, yielded a time-dependent variation of the overall geometric mean of the event count vs. fluorescence, which decreased by ~30% over the course of an hour (Figure 1). The geometric mean was plotted against incubation time and fit to a linear function prior to plotting the slope (Figure S2). Quantitative measurement of [2Fe-2S](GS)₄²⁻ cluster transport into Atm1p-embedded proteoliposomes yielded an observed rate constant of $0.06 \pm 0.01 \text{ min}^{-1}$, in good agreement with the transport kinetics determined for other ABC transporter, such as OpuA.¹⁵ The corresponding control experiments without ATP or Atm1p showed a relatively unchanged geometric mean, indicating that the overall fluorescence of the proteoliposome only decreases in the presence of the transporter protein Atm1p and the cofactor Mg-ATP.

During proteoliposome synthesis, the phospholipids were resuspended in the presence of 1 mM fluorescein, which encapsulates the fluorophore within the liposome. Subsequent insertion of Atm1p is also performed with excess fluorescein present in the solution to prevent diffusion during the addition of detergent. The final product contains the fluorescent probes and therefore can be readily quantified by flow cytometry. An interesting phenomenon was observed for the GSH only, and GSH/S²⁻ control experiments, where the rate constants are non-zero, but significantly slower than that of the cluster experiment. This

agrees with prior observations from our laboratory²⁰ and others,²¹ where glutathione alone was also found to stimulate ATPase activity, and may be transported weakly. Additional ferric and sulfide ions did not provide any significant increase in fluorescence signal change.

Complementary studies were also carried out by monitoring the transport of fluorescein-labeled glutathione cluster into a non-fluorescent proteoliposome. Synthesis and characterization of the fluorescein-labeled glutathione Fe-S cluster are detailed in the SI. Binding of the labeled cluster to Atm1p was assessed by titration of fluorescein-labeled cluster to Atm1p (Figure S3) to yield an observed $K_D = 118 \pm 11 \mu\text{M}$, which compares favorably with the $68 \pm 2 \mu\text{M}$ determined for the unlabeled cluster by inhibition of ATPase activity,²⁰ and demonstrating that the labeled cluster can orient in the substrate pocket without significant steric interference. The resulting fluorescein-labeled cluster was used to assay cluster transport into the proteoliposome, and the final proteoliposome product (containing labeled cluster) was detected by flow cytometry methods (Figure 2). Over the incubation period of 1 h, sufficient numbers of fluorescein-labeled clusters were taken up to yield the kinetic profile for proteoliposome transport by Atm1p.

Flow cytometry experiments were further complemented by use of tiron-iron complex formation in a colorimetric assay. Tiron yields a higher extinction coefficient in the visible range relative to other iron chelators such as BPTD and EDTA.²⁴ Following cluster transport across the membrane bilayer, the concentration of iron was readily and independently quantified following release by acidification of the proteoliposome product with concentrated HCl. The product was then neutralized by addition of NaOH and buffered with MES for tiron chelation detection, a complexing agent selective for ferric ions.²⁵ A limiting factor for cluster uptake is the finite size and volume of the liposome.¹⁵ However, after 1 h incubation, significantly higher ferric ion concentrations were detected inside the proteoliposome, relative to control experiments carried out in the absence of protein and Mg-ATP (Figure 3), and the time-dependent change in absorbance yielded an observed rate constant of $0.07 \pm 0.02 \text{ min}^{-1}$ for cluster transfer. The raw measured concentration of $3.6 \pm 0.3 \mu\text{M}$ reflects an estimated liposomal concentration of $\sim 0.3 \text{ mM}$ after correction for sample dilutions.¹⁵

A low level of iron was also observed in the control experiments for the tiron assay and this background noise was not removed by additional washing of the proteoliposomes. A rational interpretation of this observation is that a small component of the cluster may become interstitially trapped in the membrane bilayer, and is then solubilized along with the transported cluster and contributes to the overall iron concentration. Background signal was also observed in cluster transport experiments that were analyzed by OES-MS, where an elevated level of iron was detected in the proteoliposome product in the absence of the transporter protein.²⁶ This explanation is also supported by the control experiments from the flow cytometry data, where no significant amount of signal change was observed without the essential components of the reaction. It is significant that essentially no transported iron was detected in controls that used free iron ion rather than cluster, and so neither transported nor background-associated iron appear to stem from any product of cluster degradation in the reaction solution (and in fact no degradation would be expected under the experimental conditions used).

Conclusions

In summary, the results support a model (Figure S4) for ABCB7-type transporters that uses glutathione-coordinated iron sulfur cluster as the physiological substrate for mitochondrial cluster export to the cytosol. The $[2\text{Fe-2S}](\text{GS})_4^{2-}$ cluster demonstrates a two-fold greater stimulation of ATPase activity at concentrations three orders of magnitude lower than that required for stimulation by glutathione alone,²⁰ and most likely the association and stimulation by free glutathione represents partial population of the site occupied by $[2\text{Fe-2S}](\text{GS})_4^{2-}$. This also provides a rationale for the likely transport of glutathione-coordinated heavy metals,¹⁷ which most likely mimic the $[2\text{Fe-2S}]$ core. Results from electronic absorption studies support the independent findings from flow cytometry experiments, and quantitative measurement of substrate transport rates into Atm1p-embedded proteoliposomes yielded an observed rate constant for $[2\text{Fe-2S}](\text{GS})_4^{2-}$ cluster transport from the independent colorimetric assays of $0.07 \pm 0.02 \text{ min}^{-1}$, in good agreement with the $0.06 \pm 0.01 \text{ min}^{-1}$ obtained from flow cytometry. The results of this study not only support the glutathione-complexed $[2\text{Fe-2S}]$ cluster as a natural substrate for ABCB7-type mitochondrial transporters, but also demonstrate a quantitative methodology for study of metal translocation proteins and their proteoliposome products that will be invaluable in ongoing quantitative studies of metal cofactor transport for iron-sulfur clusters and other metal cofactor species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig.1.

Atm1p-mediated cluster transport into fluorescein-loaded proteoliposome is demonstrated by incubation in the presence of Mg-ATP. The fluorescent response is measured by flow cytometry at 530 nm, and the geometric mean of the signal indicates a decreasing kinetic profile for fluorescein within the proteoliposome as a result of the inner filter effect from [2Fe-2S] cluster, which partially absorbs the fluorescein signal within the proteoliposome. Cluster transport, along with control experiments conducted in the absence of Atm1p and Mg-ATP are denoted in black, red, and green, respectively. Controls with GSH only, GSH + Fe^{3+} , and GSH + S^{2-} are shown in Figure S2.

Fig. 2.

Fluorescence flow cytometry measurements of the proteoliposomes incubated with Mg-ATP (12 μ M) and fluorescein-labeled glutathione [2Fe-2S] cluster (1 mM) after 1 hr at r.t. and pH 7.5, with Atm1p (left), and without Atm1p (right).

Fig.3.

Concentration of iron inside the proteoliposome following 1 hr incubation with Mg-ATP and cluster. The concentration of iron was quantified by tiron coordination and comparison of absorbance values to a standard calibration curve. The final concentrations represent the results from diluted samples following work up of the liposome samples. Data, such as that shown in this plot is intended to compare relative concentrations, rather than absolute values. In fact, the absolute concentrations will be higher, but can only be estimated based on the inner proteoliposome volume. According to Geertsma et al.,¹⁵ the total internal volume is estimated to be ~ 5 μ L, and the corresponding concentration of the glutathione Fe-S cluster inside the liposome after the transport experiment is therefore estimated to be ~ 0.3 mM.